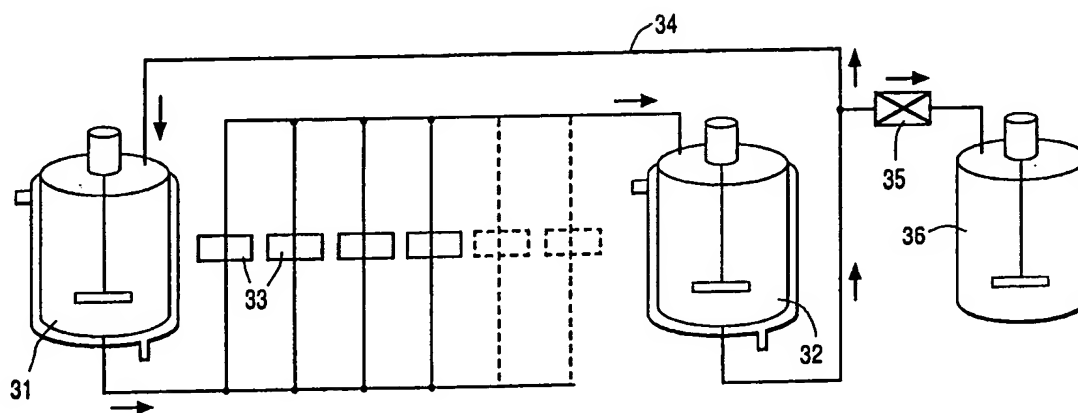




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US91/07272 <b>(22) International Filing Date:</b> 4 October 1991 (04.10.91)  <b>(30) Priority data:</b> 593,200                      5 October 1990 (05.10.90)      US  <b>(71) Applicant:</b> THE LIPOSOME COMPANY, INC. [US/US]; One Research Way, Princeton Forrestal Center, Princeton, NJ 08540 (US).  <b>(72) Inventors:</b> COE, Royden, M. ; 17 Hanover Court, Borden-town, NJ 08505 (US). THIES, Robert, L. ; 2241 Dunbar Street, Vancouver, British Columbia V6R 3M8 (CA). PORTNOFF, Joel, B. ; 76 Gregory Place, Richboro, PA 18954 (US).		<b>(74) Agent:</b> BLOOM, Allen: The Liposome Company, Inc., One Research Way, Princeton Forrestal Center, Princeton, NJ 08540 (US).  <b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report.          Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>

(54) Title: LIPOSOME EXTRUSION PROCESS



## (57) Abstract

A method of sizing liposomes by passing a suspension of liposomes through an aluminum oxide porous film under pressure is disclosed. In a preferred embodiment, the porous film is a branched type anodic aluminum oxide porous film. The process produces a population of liposomes substantially free of liposomes above a pre-determined maximum size useful in administering drugs or other bioactive agents, such as in the treatment of cancer or AIDS-related illnesses. Also disclosed for carrying out the method is an apparatus comprising one or more filter assemblies for holding the aluminum oxide porous film in operational configuration (33), a pressurized supply vessel for supplying the suspension of liposomes under pressure to filter assemblies (31), and a receiving vessel for receiving the filtrate from the filter assemblies (32).

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- 1 -

## LIPOSOME EXTRUSION PROCESS

This invention relates to a method of sizing liposomes, and more particularly to a sizing method which includes extruding liposomes through a branched-pore type aluminum oxide porous film.

Liposomes are completely closed lipid bilayer membranes containing an entrapped aqueous volume. Liposomes may be unilamellar vesicles (possessing a single bilayer membrane) or multilamellar vesicles (onion-like structures characterized by multiple membrane bilayers, each separated from the next by an aqueous layer). The bilayer is composed of two lipid monolayers having a hydrophobic "tail" region and a hydrophilic "head" region. The structure of the membrane bilayer is such that the hydrophobic (nonpolar) "tails" of the lipid monolayers orient toward the center of the bilayer while the hydrophilic "head" orient towards the aqueous phase.

The original liposome preparation of Bangham, et al. (J. Mol. Biol., 1965, 13:238-252) involves suspending phospholipids in an organic solvent which is then evaporated to dryness leaving a phospholipid film on the reaction vessel. Next, an appropriate amount of aqueous phase is added, the mixture is allowed to "swell", and the resulting liposomes, which consist of multilamellar vesicles (MLVs), are dispersed by mechanical means. This technique provided the basis for the development of the small sonicated unilamellar vesicles (SUVs) described by Papahadjopoulos et al. (Biochim. Biophys. Acta., 1968, 135:624-638), as well as large unilamellar vesicles (LUVs). In addition, U.S. Patent No. 4,235,871, issued November 25, 1980 to Papahadjopoulos et al., describes a "reverse-phase evaporation process" for making oligolamellar lipid vesicles, also known as reverse-phase evaporation vesicles (REVs).

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- 2 -

Alternative methods have been developed for forming improved classes of multilamellar vesicles which have been shown to have particularly improved properties such as, for example, higher active ingredient trapping efficiencies and loadability, better stability, less leakage, and greater ease of production. One such improved class of liposomes, denominated as stable plurilamellar vesicles (SPLVs), is described in U.S. Patent No. 4,522,803, issued June 11, 1985 to Lenk et al. Another such improved class, defined as monophasic vesicles (MPVs), is described in U.S. Patent No. 4,558,578, issued May 13, 1986 to Fountain et al. Both of these classes of liposomes have also been characterized as having substantially equal interlamellar solute distributions. A general review of various methods for producing liposomes, including an extensive bibliography, is set forth in Deamer and Uster, "Liposome Preparation: Methods and Mechanisms", in the Liposomes, edited by M. Ostro, pp. 27-51 (1983), incorporated herein by reference.

The administration of drugs encapsulated in or otherwise associated with liposomes has been proposed for use in a variety of drug delivery regimens in combination with or as an alternative to the administration of free drugs. In some applications, liposomes have been found to provide sustained release of drugs for extended periods, which can be of particular importance in the lengthy chemotherapy regimens often required for the treatment of various forms of cancer or AIDS-related illnesses. Another property of liposomes is their ability to be taken up by certain cells, such as phagocytes, such that they can deliver their active ingredient to the interior of the cells. This makes such liposome treatment particularly useful in treating intracellular infections, such as those associated with species of Mycobacteria, Brucella, Listeria, and Salmonella. Thus, drugs encapsulated in liposomes can be delivered for the treatment of such intracellular diseases without administering large amounts of free unencapsulated drug into the bloodstream. In addition, the mere association of certain drugs or

- 3 -

other bio-active agents with liposomes has been found to potentiate or improve the activity of such drugs or bio-active agents, or to reduce their toxicity.

5       Liposomes behave like particles, and are commonly described in terms of average particle size and particle-size distributions. For certain uses of liposomes, particularly in the parenteral administration of drugs, it is important to size the liposomes to a  
10       desired average particle size, and to maintain a controlled particle-size distribution, particularly by sizing the liposomes so that substantially all of the liposomes are of a size below a predetermined maximum diameter. For liposomes intended for  
15       parenteral administration, one desirable size range is between about 100 and 1000 nm, preferably between about 100 and 500 nm. (As used herein, nm represents nanometer ( $10^{-9}$  m) and um represents  
20       micrometer or micron ( $10^{-6}$  m).) The maximum desired size range is often limited by the desire to sterilize the liposomes by filtering through conventional sterilization filters, which commonly have a  
25       particle-size discrimination of about 200 nm. However, overriding biological efficacy and/or safety factors may dictate the need for a particular particle size, either larger or smaller. Control of the size range of the liposomes may also improve the effectiveness of the liposomes in vivo, as well as the stability and leakage resistance of the liposomes.

      The various methods for producing liposomes generally produce a suspension of liposomes of widely varying sizes, many of which exceed 1000 nm in average particle size. A number of methods have  
30       been proposed to reduce the size and size distribution of liposomes in such suspensions. In a simple homogenization method, a suspension of liposomes is repeatedly pumped under high pressure through a small orifice or reaction chamber until a desired average size of liposome particles is achieved. A limitation of this method  
35       is that the liposome size distribution is typically quite broad and variable, depending on the number of homogenization cycles, pressures, and internal temperature.

- 4 -

Small unilamellar vesicles (SUVs), generally characterized as having diameters below 100 nm, are composed of highly strained, curved bilayers. The SUVs are typically produced by disrupting larger liposomes via ultrasonication. It has been found that a narrow size distribution of such liposomes can only be achieved when the liposomes have been reduced to their smallest sizes, less than about 50 nm. Furthermore, this process may not be amenable to large-scale production, because it is generally conducted as a batch process with long-term sonication of relatively small volumes. In addition, heat build-up during sonication can lead to peroxidative damage to lipids, and sonication probes may shed titanium particles which are potentially quite toxic in vivo.

A method of sizing liposomes by filtration through a 200-nm Unipore<sup>TM</sup> polycarbonate filter is discussed in Szoka, Proc. Natl. Acad. Sci. U.S.A., 75:4194-8 (1978). A size-processing method based on liposome extrusion through a series of uniform straight-pore type polycarbonate membranes from about 1000 nm down to about 100 nm is described in Hunt et al., U.S. Patent No. 4,529,561, issued July 16, 1985. However, this method can be relatively slow, often requiring many passes through various size filters to obtain the desired particle-size distribution.

Vesicles may also be size-reduced using an extrusion process described in Cullis et al., U.S. Patent No. 5,008,050, issued April 16, 1991, incorporated herein by reference. Vesicles made by this technique are extruded under pressure through a filter with a pore size of 100 nm or less. This procedure avoids the problems of the above homogenization and sonication methods, and does not require multiple passes through decreasing size filters, as described in the above-cited U.S. Patent No. 4,529,561. Such a process can provide size distributions of liposomes that are quite narrow, particularly by cycling the material through the selected size filter several times. In addition, it is believed that such extrusions may convert

- 5 -

multilamellar vesicles into oligolamellar or even unilamellar form, which may be desired for certain applications. However, as demonstrated by the Examples set forth below in the present specification, when such extrusions are made through 100-nm polycarbonate filters, such as the Nuclepore<sup>TM</sup> filters used in the examples of this reference, even at relatively high pressures flow rates may be relatively low.

U.S. Patent No. 4,737,323, issued April 12, 1988, describes a method for sizing liposomes by extrusion through an asymmetric ceramic filter. Such filters are designed for operation at relatively high pressure, and can be backflushed to prevent clogging. U.S. Patent No. 4,927,637, issued May 23, 1990 describes a method of sizing liposomes by passing them through a polymer filter having a web-like "tortuous-path" construction.

An alternative type of filter medium is described in Furneaux et al., U.S. Patent No. 4,687,551, issued August 18, 1987. This patent discloses a new type of filter sheet comprising an anodic aluminum oxide film having branched pores extending from one surface of the film to the other. The film is unique in that it includes a system of larger pores extending in from one face and a system of smaller pores extending in from the other face. The system of larger pores interconnects with the system of smaller pores such that the inner ends of one or more smaller pores are joined to the inner end of a larger pore and there are substantially no blind larger pores. This patent is incorporated by reference into the present specification for the purpose of disclosing such branched-pore type aluminum oxide porous films and the method for forming them.

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- 6 -

In a particular embodiment, the branched-pore anodic aluminum oxide film of the Furneaux et al. patent is described as:

An anodic aluminum oxide film having pores extending from one face of the film to the other,

including a system of larger pores extending in from one face a distance into the film, the larger pores having a diameter  $d$  near their inner ends, and a system of smaller pores extending in from the other face a distance  $s$  into the film, the smaller pores having a substantially uniform minimum diameter  $p$ ,

the system of larger pores interconnecting with the system of smaller pores, such that the inner ends of one or more smaller pores are joined to the inner end of a larger pore and there are substantially no blind larger pores, wherein

$d$  is 10 nm to 2  $\mu\text{m}$

$p$  is at least 2 nm but less than 0.5  $d$ , and

$s$  is 10 nm to 1  $\mu\text{m}$ .

The size rating of such branched-pore type films is equal to  $p$ , the substantially uniform minimum diameter of the smaller pores.

Filtration membranes made in accordance with the disclosure of the Furneaux et al. patent are commercially available and sold by the Anotec Separations, New York, NY, under the name Anopore<sup>TM</sup>. Additional information regarding such branched-pore type membranes is provided in Furneaux et al., "The Formation of Controlled-Porosity Membranes from Anodically Oxidized Aluminum", Nature 337:147-9 (1989).

One use of such branched-pore Anopore<sup>TM</sup> filters is described in Jones et al., "Comparison of a New Inorganic Membrane Filter (Anopore) with a Track-Etched Polycarbonate Membrane Filter (Nuclepore) for Direct Counting of Bacteria", Applied and

- 7 -

Environmental Microbiology 55(2):529-30 (1989). This article compares the bacteria filtering ability of a 200-nm-pore-size Anopore<sup>TM</sup> filter against a 200-nm-pore-size Nuclepore<sup>TM</sup> filter.

5 In accordance with the method of the present invention, a population of liposomes substantially free of liposomes above a predetermined maximum size is produced by (1) providing a suspension of liposomes, a portion of which are of sizes larger than the  
10 predetermined maximum size; and (2) passing the suspension under pressure one or more times through an aluminum oxide porous film.

Films with a pore size of 1000 nm or less may be used to obtain liposomes with an average particle size of in the range of about 100 to 1000 nm. In a particular embodiment of the present invention, a  
15 film with a pore-size rating of 200 nm or less is used to obtain a population of liposomes with a predetermined maximum diameter of less than about 500 nm. In another embodiment, a film with a pore size of about 100 nm or less is used, and the suspension of liposomes is passed through the filter one or more times until the average  
20 liposome particle size is about 100 to 200 nm.

In a further embodiment of the present invention, the suspension of liposomes is passed repeatedly through the porous film until a desired particle size distribution is obtained. In a  
25 particular embodiment, the liposomes are passed through the porous film two to ten times. In an additional embodiment, the liposomes are presized by being passed one or more times through a 2-10 micrometer filter.

30 A preferred film for use in the present invention is a branched-pore type anodic aluminum oxide porous film. As discussed above, such a branched-pore anodic aluminum oxide porous film is an aluminum oxide sheet having two substantially parallel major faces with pores extending from one face of the sheet to the other,  
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- 8 -

including a system of larger pores extending from one face into the sheet and a system of smaller pores extending in from the other face, the system of larger pores interconnecting with the system of smaller pores such that the inner ends of one or more smaller pores are joined to the inner end of a larger pore and there are substantially no blind larger pores. The size rating of such branched-pore type films is equal to the minimum diameter of the smaller pores, which are preferably substantially uniform.

In a further embodiment of the present invention, apparatus is provided for carrying out the filtration method. The apparatus comprises one or more filter assemblies for holding the aluminum oxide porous films in operational configuration, means for supplying the suspension of liposomes to the filter assemblies, and means to receive the filtrate from the assemblies. In a particular embodiment, two or more assemblies are used in parallel configuration to filter the liposome suspension passing from the supply means to the receiving means. Optionally, means can also be provided for recirculating at least a portion of the filtrate from the receiving means back to the supply means, thus providing for multiple passes of the liposomes through the filters. In addition, a sterilization filter can be provided downstream of the receiving means, as may be appropriate to prepare the filtrate for pharmaceutical use.

The extrusion is rapid and inexpensive, and does not require the use of solvents or other chemicals that must be removed.

Figure 1 is a simplified top view, not to scale, of a filter assembly made in accordance with the present invention.

Figure 2 is a simplified cross-sectional side view, not to scale, through line 2-2 of Figure 1, of the filter assembly shown in Figure 1.

- 9 -

Figure 3 is a schematic representation of an extrusion system made in accordance with the present invention.

5 As discussed in the Furneaux et al. references cited above, aluminum can be anodized in acid to produce a uniform array of cells or openings having cylindrical pores which preferably branch from larger pore-size openings in one face of the film to smaller pore-size openings in the other face of the film. Such filters are available in a variety of pore sizes, and ones having a pore size of 10 the smaller pores of less than about 1000 nm are preferred for use in the present invention, although smaller or larger pore-sizes can be used depending on the final application of the liposomes. (Hereinafter, unless otherwise noted, "pore size" for such filters shall refer to the minimum pore size of the smaller pores.) At 15 present, of the Anopore<sup>TM</sup> anodized aluminum porous filters commercially available from Anotec Separations, those of pore size under 200 nm are of the branched-pore structure, and those of pore size of 200 nm or larger are of uniform pore size from one surface to the other. When the desired average particle size of the 20 liposomes is less than about 200 nm, then the preferred pore size of the filter is less than about 100 nm. These filters are also of the preferred branched-pore type structure.

25 These aluminum oxide filters are hydrophilic; they do not swell in aqueous solvents; they have good organic solvent resistance; and they have pores of uniform size which promote high flow-through characteristics. Because of the properties of such films, it was found that liposomes could be extruded through them at relatively high flow rates under relatively low pressure (see Example 3, 30 below). Thus, aluminum oxide filters are shown to be superior for extruding liposomes over the previously known polymeric filters.

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- 10 -

In the present invention, the branched-pore filters are preferably used so that the liposomes enter the face with the smaller size pores and exit through the face with the larger size pores. However, as shown in the examples below, good extrusion can  
5 also be obtained with a filter mounted in the inverted position, so that the liposomes enter the large pore-size face.

In accordance with the process of present invention, a  
10 population of liposomes substantially free of liposomes above a predetermined maximum size is produced from a suspension of liposomes, a portion of which are of sizes larger than the predetermined maximum size. The process includes passing the suspension of liposomes under pressure one or more times through an  
15 aluminum oxide porous film, such as one of the type described above.

To determine whether a population of liposomes is "substantially free of liposomes above a predetermined maximum size", the liposomes can be tested using a standard sizer. One such  
20 standard sizer is a Malvern Sizer, available from Malvern Instruments, Malvern, England, which is described and used in some of the examples below. Another sizer which can be used to determine particle size distributions is a Nicomp<sup>TM</sup> laser particle sizer, available from Hiac/Royco Instruments, Menlo Park, CA, which is also  
25 described and used in some of the examples below. In the particle size distributions reported in the examples below, a test result indicating that 0.0 percent of the liposomes present in the population are above a given size indicates that the population is "substantially free" of such large-size liposomes.

30 Although not required, the particle size, and particularly the particle-size distribution, of liposomes may be made smaller and more uniform by extruding through a larger filter as a first step. For example, extruding the liposomes through a suitable filter of  
35 2-10 micrometer size, such as one made from polytetrafluoroethylene

- 11 -

(PTFE), as is well known in the art, will reduce the particle size and particle-size distribution prior to extruding through the aluminum oxide filter, and may thereby reduce the time for extrusion.

5

The pressure during the extrusion will be varied depending upon the liposomes employed, their mean particle diameter and particle-size distribution, and the rate of flow desired. Extrusion pressures may vary from about 200 to about 1000 psi (1.4-6.9 MPa), but pressures of less than about 600 psi (4.2 MPa) are preferred.

10

In general, fewer extrusion passes are required when using the branched-pore type aluminum oxide filters of the present invention, as opposed to the previously known polycarbonate filters, for similar results in terms of particle size, particle-size distribution and flow rates. However, repeated extrusion passes through the aluminum oxide filters may be used to obtain a narrower particle-size distribution, and particularly to reduce all liposomes to below a predetermined maximum size. For example, 2-10 extrusions of the liposomes through the filters are preferred to decrease the particle-size distribution, thereby producing relatively uniform liposomes of high capacity in a rapid, efficient and inexpensive manner. Multiple extrusions may also convert multilamellar vesicles to more desired oligolamellar or unilamellar forms.

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Subsequent to the extrusion process of the invention, any free unencapsulated therapeutic agent or other solution can be readily removed, as by dialysis or diafiltration, leaving stable drug encapsulating liposomes of relatively uniform size. The resultant liposomes may be readily measured into uniform dosages for administration parenterally or orally.

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The invention will be further illustrated by the following examples, but the invention is not meant to be limited to the details described therein.

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- 12 -

## EXAMPLE 1

Preparation of Liposomes

5 Three batches of liposomes (hereinafter designated A, B and C) were prepared as follows:  
71.3 mg/ml egg phosphatidylcholine (obtained from Princeton Lipids, Princeton, New Jersey) and 28.7 mg/ml cholesterol (J.T. Baker, Phillipsburg, New Jersey) were dissolved in 0.15 to 0.5 ml of  
10 methylene chloride and added to a 300-mM citrate buffer solution (pH 4.0) to make up a 1-ml volume. The methylene chloride was removed by heating the mixture to about 40°C. To aid in the removal of the solvent, nitrogen was sparged through Batches A and C, while Batch B was heated under partial vacuum.

15 The resultant liposomes were vesicles of various sizes and various size distributions. The initial size distributions of these liposomes prior to size reduction were measured on a Malvern Sizer 3600 E Type with a 63-mm lens, available from Malvern Instruments, Malvern, England. The results are presented in Table I, in which  
20 the mean diameters and size distribution ranges are expressed in micrometers (um). Before extrusion, Batch B was presized through a 5-um pore-size Mitex<sup>TM</sup> PTFE filter (Millipore Corp., Bedford, Mass.), and the mean diameter and distribution range for the Batch B liposomes after presizing is included in the table. Batches A and C  
25 were not put through presizing. The results show a considerable batch-to-batch variation in the size distribution of the unsized liposomes.

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- 13 -

TABLE I  
Liposome Sizes Prior to Extrusion

	<u>Batch</u>	<u>Mean Diameter (um)</u>	<u>Distribution Range (um)</u>
5	A	23.2	1.5-118
	B	14.9	1.5-118
	B*	2.5	<1.2-5
10	C	3.3	<1.2-14

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\* After 5 micrometer presizing

EXAMPLE 2  
Extrusion of Liposomes

15

In accordance with the present invention, the liposomes of Example 1 were extruded five times under pressure through an Anopore<sup>TM</sup> 90-mm diameter, 100-nm pore size (small pores) branched-pore aluminum oxide filter of the type described above.

20 The placement of the filter for Batches A and B, was with the input through the small-pore surface. Good results were also obtained for Batch C, which was extruded with the filter inverted so that the input was through the large-pore surface. The mean diameter of the liposomes and the particle-size distributions of the liposomes were

25 measured after the indicated passes through the filter. The size distributions were measured on a Nicomp<sup>TM</sup> Model 370 laser particle sizer, available from Hiac/Royco Instruments, Menlo Park, CA. The results measured after each extrusion pass are summarized in Table II:

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- 14 -

TABLE II  
Mean Diameter and Particle Size Distribution  
After Each Extrusion Pass

	Pressure PSI (MPa)	Extrusion Rate Liters/min	Mean Diameter ( $\mu$ m)	<0.1 $\mu$ m percent	0.1- 0.45 $\mu$ m percent	>0.45 $\mu$ m percent
5	<u>Batch A:</u>					
	325 (2.2)	NT	NT	NT	NT	NT
		0.2	0.188	2.6	94.4	3.0
10		0.2	0.168	6.7	92.6	0.8
		0.2	0.132	7.6	92.3	0.0
		0.2	0.123	3.6	96.4	0.0
	<u>Batch B:</u>					
	500 (3.4)	0.02	0.180	19.1	78.6	2.2
	changed filter					
	300 (2.1)	1.1	0.139	6.3	73.7	0.1
		1.2	0.133	20.2	79.8	0.0
15		1.5	0.126	35.1	64.9	0.0
		1.5	0.116	36.1	63.9	0.0
	<u>Batch C:</u>					
	(inverted filter)					
	300 (2.1)	0.7	0.665	1.0	48.3	50.6
		0.7	0.162	23.2	76.2	0.6
		0.7	0.143	26.5	73.3	0.1
		0.3	0.149	22.5	77.5	0.0
20	325 (2.2)	0.5	0.144	23.6	76.3	0.0

NT - not tested

## COMPARATIVE EXAMPLE

25

For comparison, a sample of the liposomes prepared in Batch C of Example I was extruded through a total of eight passes, first five times through a 90-mm diameter, 200-nm pore size Nuclepore<sup>TM</sup> polycarbonate filters, two times through a 100-nm Nuclepore<sup>TM</sup> filter, and once through a 220-nm sterilization filter.

30

(Nuclepore<sup>TM</sup> filters are commercially available from Nuclepore, Inc., Pleasanton, CA.) A second sample was extruded in four passes through a 90-mm diameter, 100-nm pore size Anopore<sup>TM</sup> filter, in accordance with the present invention. As with the first sample,

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- 15 -

this sample was then passed through a 220-nm sterilization filter. Size data was measured after the pass numbers indicated in the first column. All of the extrusions were conducted at the same pressure of 400 psi (2.8 MPa). The results are presented in Table III:

5

TABLE III

Mean Diameter and Particle Size Distribution after Extrusion

	Pass Number	Pressure PSI (MPa)	Extrusion Rate Liters/min	Mean Diameter ( $\mu$ m)	<0.1 $\mu$ m Percent	0.1- 0.45 $\mu$ m Percent	>0.45 $\mu$ m Percent
<u>Sample 1:</u>							
10	200-nm Nuclepore						
	5	400 (2.8)	0.7	0.195	8.5	90.1	1.4
	100-nm Nuclepore						
	6	400	0.3	0.168	13.5	86.3	0.3
15	7	400	0.2	NT	NT	NT	NT
	220-nm sterile filter						
	8		NT	0.152	4.0	96.1	0.0
<u>Sample 2:</u>							
20	100-nm Anopore						
	2	400 (2.8)	0.7	0.174	22.0	76.9	1.1
	4	400	0.8	0.146	17.5	82.5	0.0
	220-nm sterile filter						
	5		NT	0.150	12.0	88.0	0.0
NT - not tested							

The branched-pore type aluminum oxide filters of the present invention required fewer passes with a higher flow rate than the polycarbonate filters to obtain a similar particle-size distribution.

## EXAMPLE 3

An additional test was conducted to compare the size-reduction capabilities of a branched-pore type anodized aluminum oxide film with an equivalent pore-sized polycarbonate filter. The tests were performed using egg phosphatidylcholine and cholesterol liposomes, made in accordance with Example 1, with the liposomes in aqueous

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- 16 -

suspension at 100 mg/ml. For this example, the cholesterol was obtained from Croda Chemicals, New York, New York. The initial size distribution, as measured on the Malvern Sizer, showed a median diameter of 10.9  $\mu\text{m}$ , and a range of diameters of 2.4 to 118  $\mu\text{m}$ . To facilitate submicron size reduction, the batch was processed twice through a 5- $\mu\text{m}$  Mitex<sup>TM</sup> PTFE filter at a pressure of 100 psi (0.7 MPa). After this step, the median diameter of the liposomes was measured as 3.5  $\mu\text{m}$ , and the range of diameters was 1.9 to 11  $\mu\text{m}$ .

The batch was divided into two portions. Portion number one was extruded through a 0.1 micrometer Anopore<sup>TM</sup> filter, and portion two was extruded through a 0.1 micrometer Nuclepore<sup>TM</sup> polycarbonate filter. The starting extrusion pressure for both portions was 300 psi (2.1 MPa). The extrusion flow rate, particle size, and particle size distribution were measured for each pass through the filters. A total of five passes were performed on each portion. The results are presented in Table IV:

TABLE IV

Mean Diameter and Particle Size Distribution after Extrusion

Pass Number	Pressure PSI (MPa)	Extrusion Rate Liters/min	Mean Diameter ( $\mu\text{m}$ )	<0.1 $\mu\text{m}$ Percent	0.1-0.45 $\mu\text{m}$ Percent	>0.45 $\mu\text{m}$ Percent
<u>Portion 1 (Anopore 100-nm filter):</u>						
1	300 (2.1)	0.6	0.565	0.0	38.6	61.4
2	300	0.7	0.212	10.5	85.8	3.7
3	300	0.8	0.186	10.5	88.7	0.8
4	300	0.8	0.172	3.6	96.4	0.0
5	300	0.8	0.165	10.7	89.3	0.0
<u>Portion 2 (Nuclepore 100-nm filter):</u>						
1	300 (2.1)-	0.02	1.390	0.0	17.6	82.4
..	500 (3.4)*					
2	700 (4.8)	0.2	0.215	11.2	84.9	4.0
3	700	0.2	0.178	6.5	93.2	0.3
4	700	0.2	0.167	7.4	92.6	0.0
5	700	0.3	0.158	19.1	80.9	0.0

\* - Pressure increased from 300 to 500 psi after 65% filtered.

- 17 -

These data demonstrate that both filters are capable of producing similar size distributions with the same number of passes. However, the aluminum oxide filter used for the first portion required less pressure and operated at a much higher flow rate than the polycarbonate filter used for the second portion. All of the passes through the aluminum oxide filter were conducted at 300 psi (2.1 MPa), with flow rates of 0.6 - 0.8 liters/min. When the extrusion was repeated using the polycarbonate filter, the initial pressure had to be increased from 300 psi (2.1 MPa) to 500 psi (3.4 MPa) just to complete the first pass through the filter at a very low flow rate of 0.02 liter/min. For passes 2 through 5, a higher pressure of 700 psi (4.8 MPa) was needed to maintain a flow rate of 0.2 - 0.3 liter/min.

## EXAMPLE 4

A further test was conducted to study the differences in the extrusion properties of liposomes with respect to the orientation of the 100 nm Anopore<sup>TM</sup> branched-pore filter used to size reduce the liposomes. As discussed above, the Anopore 0.1 um branched-pore filter has a small-pore side, having 100 nm pores, and a large-pore side, having 200 nm pores. In this test, a comparison was made to determine the effects of passing aliquots of the same liposome material through the 100 nm Anopore filters with the small-pore side upstream or with the large-pore side upstream.

The test was performed using egg phosphatidylcholine and cholesterol liposomes, made in accordance with Example 1, with the liposomes in aqueous suspension at 100 mg/ml. The material was prepared in a single five liter lot, mixed well, and divided into four 750 mL samples (A-D). Samples A and B were extruded using the 100-nm upstream orientation, and samples C and D used the 200-nm upstream orientation. Extrusion of the liposomes was carried out by passing them twice through the branched-pore filters, under 400 psig (2.8 MPa) pressure.

- 18 -

The particle size distributions of the filtered materials from each of the test samples were measured using a Nicomp<sup>TM</sup> sizer, as described in Example 2, and found to be generally equivalent.

5 However, the time required to size reduce the liposomes was significantly less for samples A and B, with the 100-nm filter side upstream, as opposed to samples C and D, with the 200-nm filter side upstream. Table V presents the mean particle size diameter in nanometers (nm) and the filtration time in minutes (min):

10

TABLE V

Mean Diameters and Extrusion Rates by Filter Orientation

Sample	Mean Diameter (nm)			Filtration Time (min)	
	Start	Pass 1	Pass 2	Pass 1	Pass 2
15 A	199	152	138	0.95	0.85
B	199	151	131	0.52	0.53
C	199	157	139	4.93	1.03
D	199	169	153	3.08	0.97

20 The extruded material was then sterile filtered through a 220-nm sterilization filter, of the same type used in the Comparative Example. The sterilization filter used in this test, and in the above Comparative Example, was a commercially available Millipak<sup>TM</sup> 200 filter supplied by Millipore Corp., Bedford, Mass, and described as having a Durapore<sup>TM</sup> polyvinylidene difluoride (PVDF) tortuous path membrane. Sterile filtration was considered

25 complete when all of the material had passed through the sterilization filter, or when the steady stream of material had broken into a slow drip. The mean particle diameter (nm), the time required to pass through the filter (min), and the percent volume of

30 material which passed through the sterilization filter were measured for each sample, and the results are presented in Table VI:

35

- 19 -

TABLE VI

Effect of Extrusion Filter Orientation on  
Subsequent Sterile Filtration

Sample	Mean Diameter (nm)	Filtration Time (min)	Percent Through Filter
A	133	2.67	96 %
B	126	0.68	100 %
C	131	1.95	47 %
D	151	1.27	44 %

These data demonstrate that the sterile filtration was very efficient for samples A and B, in which almost all of the material successfully passed through the sterilization filter. In contrast, only about half of the volume of samples C and D was able to pass through the sterilization filter before the flow stopped.

## EXAMPLE 5

Figure 1 is a simplified top view, not to scale, of a filter assembly (10) made in accordance with a particular embodiment of the present invention, designed for use with a 90-mm diameter Anopore<sup>TM</sup> filter. It should also be recognized that this filter assembly could also be used to house other types of filters as well, such as, for example, the Nuclepore<sup>TM</sup> filters used in the comparative tests in Example III above. Figure 2 is a simplified cross-sectional side view, not to scale, of filter assembly (10) cut along line 2-2 of Figure 1. Filter assembly (10) comprises a filter housing top half (11) and a filter housing bottom half (12), held together by a plurality of fastening screws (13). Referring to Figure 2, filter unit (14) represents a 90-mm Anopore filter mounted on a drain disk (Nuclepore Catalog #231700) cut to 90 mm, which is in turn mounted on a 90-mm Teflon<sup>R</sup> coated mesh filter support (Millipore Catalog #YY30 090 54). Filter unit (14) is in turn mounted on a stainless steel filter support plate (15), which is

- 20 -

provided with fluid passage means, such as transverse channels (16). Support plate (11) sits into a seat portion (17) of filter housing bottom half (12), with the seat portion (17) provided with radial grooves (not shown) to channel the liquid which passes through the filter into liquid outlet (18). Filter housing top half (11) includes a ring portion (19) which holds filter unit (14) in place when top half (11) is tightened down onto bottom half (12) by screws (13).

10 In operation, the liquid to be filtered enters filter assembly (10) through liquid inlet (20), flows through filter unit (14) and filter support plate (15), and then is channeled out through filter outlet (18). Preferably, housing top half (11) is provided with a relief outlet (21), by which a relief valve (not shown) can be connected to the housing.

20 Although filter assembly (10) has been described in terms of a "top half" and a "bottom half", these references are for purposes of describing the structure, and do not reflect the orientation of the housing in operation. Because the liquid being filtered is sent to the assembly at such relatively high pressures, it is believed that the assembly can be used in any orientation.

25 Figure 3 is a schematic representation of an extrusion system made in accordance with the present invention. The liposome composition to be filtered is contained in a high pressure supply vessel (31), and the filtrate is collected in a similar receiving vessel (32). Both of these vessels are shown as being equipped with stirrers to maintain the liposome mixtures, and heat jacketed for temperature control. The liquid exits supply vessel (31) through a bottom outlet, and is carried through a one or more filter assemblies (33), of the type described above, containing 90-mm Anopore<sup>TM</sup> filters. The filter assemblies (33) are connected in parallel, with the number of such assemblies used determined by the

- 21 -

desired total flow rate from supply vessel (31) to receiving vessel (32). From receiving vessel (32), the filtrate is forced through a sterilization filter (35), such as a Millipak<sup>TM</sup> 200 filter as described in Example 4 above, and is then collected in a stirred holding vessel (36). In addition, a recycle line (34) may be provided to allow a portion of the output of receiving vessel (32) to be recycled to supply vessel (31).

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed.

- 22 -

## CLAIMS

What is claimed is:

- 5 1. A method of producing a population of liposomes substantially free of liposomes above a predetermined maximum size; said method comprising:
  - 10 A. providing a suspension of liposomes, a portion of which are of sizes larger than said predetermined maximum size; and
  - B. passing the suspension under pressure one or more times through an aluminum oxide porous film.
- 15 2. The method of claim 1 for producing a population of liposomes having a predetermined maximum size of less than about 500 nm, wherein said aluminum oxide porous film has a pore size of about 200 nm or less.
- 20 3. The method of claim 1 wherein the suspension is passed through the porous film two to ten times.
- 25 4. The method of claim 1 wherein the suspension is passed through the film under a pressure of less than about 600 psi (4.2 MPa).
- 25 5. The method of claim 1 wherein said porous film is a branched-pore type anodic aluminum oxide porous film.
- 30 6. The method of claim 5 for producing a population of liposomes having a predetermined maximum size of less than about 500 nm, wherein said branched-pore type anodic aluminum oxide porous film has a pore size of about 200 nm or less.
- 35 7. The method of claim 6 wherein the suspension of liposomes is passed one or more times through the porous film until the population of liposomes has an average particle size of about 100 to about 200 nm.

- 23 -

8. The method of claim 7 wherein said pore size is about 100 nm.
9. The method of claim 7 wherein the suspension is passed through the porous film two to ten times.
10. The method of claim 7 wherein the suspension is passed through the film under a pressure of less than about 600 psi (4.2 MPa).
11. The method of claim 5 wherein the branched-pore type anodic aluminum oxide porous film comprises:  
an aluminum oxide sheet having two substantially parallel major faces with pores extending from one face of the sheet to the other,  
including a system of larger pores extending from one face into the sheet and a system of smaller pores extending in from the other face,  
the system of larger pores interconnecting with the system of smaller pores such that the inner ends of one or more smaller pores are joined to the inner end of a larger pore and there are substantially no blind larger pores.
12. The method of claim 10 for producing a population of liposomes having a predetermined maximum size of less than about 500 nm, wherein the smaller pores of said porous film have a substantially uniform minimum diameter of about 200 nm or less.
13. The method of claim 12 wherein said minimum diameter of the smaller pores is about 100 nm or less.
14. The method of claim 13 wherein the suspension of liposomes is passed one or more times through the porous film until the population of liposomes has an average particle size of about 100 to about 200 nm.

- 24 -

15. The method of claim 14 wherein the suspension of liposomes is passed through the porous film two to ten times.

16. The method of claim 1 further comprising presizing the liposomes, before said step of passing the suspension of liposomes through said porous film, said presizing comprising passing the suspension of liposomes one or more times through a filter with a pore size in the range of about 2 to 10 micrometers.

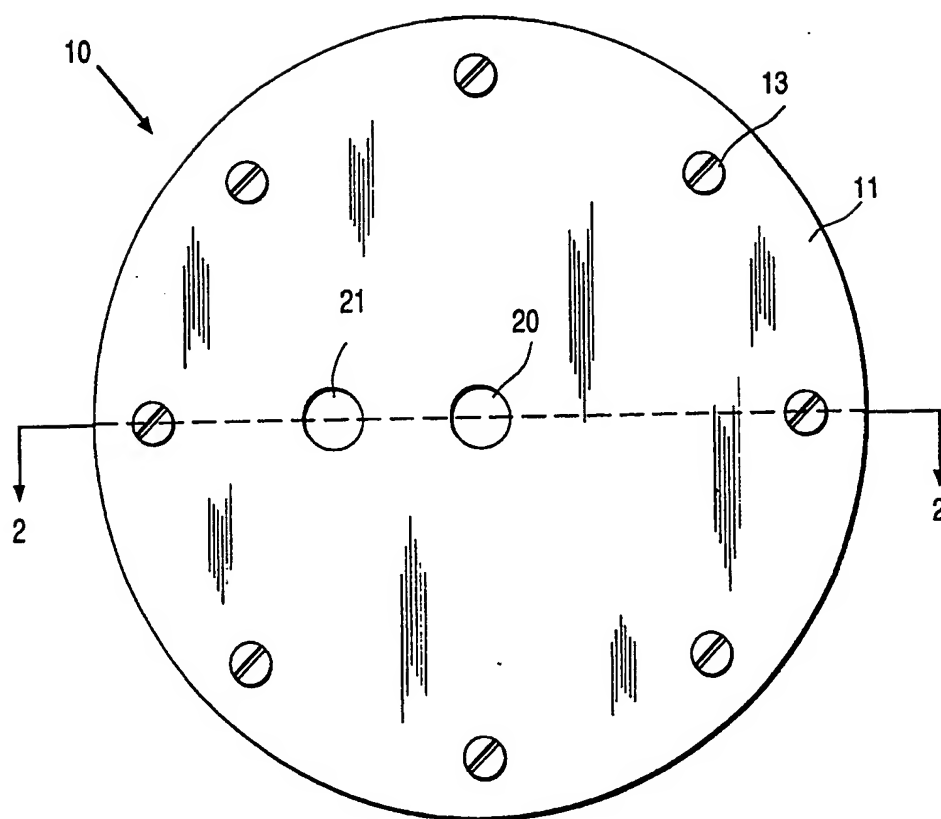
17. Apparatus for carrying out the method of claim 1, the apparatus comprising:

- A. one or more filter assemblies for holding said aluminum oxide porous film in operational configuration;
- B. pressurized supply means for supplying said suspension of liposomes under pressure to filter assemblies
- C. receiving means for receiving the filtrate from said filter assemblies.

18. The apparatus of claim 17 comprising two or more of said filter assemblies, wherein said filter assemblies are arrayed in said apparatus for operatively parallel flow between said supply means and said receiving means.

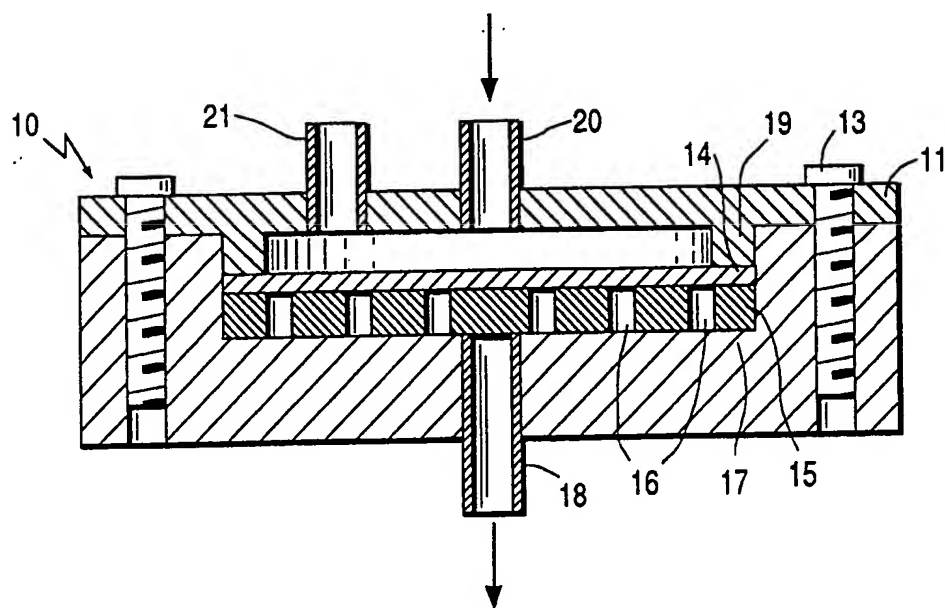
19. The apparatus of claim 17 further comprising recirculation means for recirculating filtrate from said receiving means to said supply means.

1/3

**FIG. 1**

2/3

**FIG. 2**



3/3

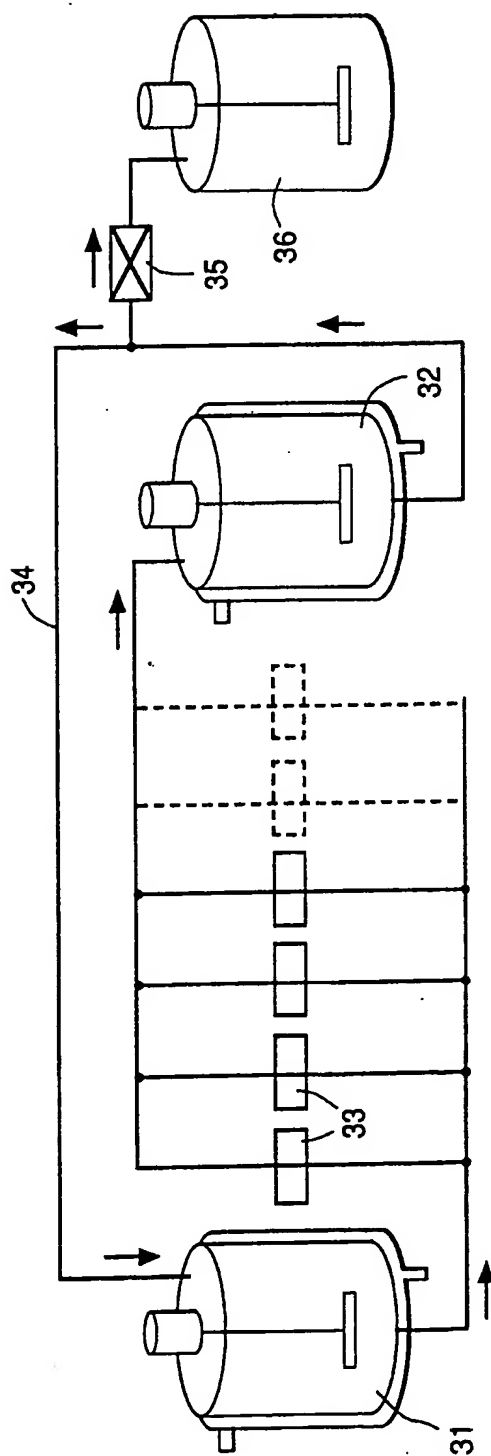


FIG. 3

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/07272

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
INT. CL.(5): A61K 9/127; B01J 13/20		
U.S. CL.: 264/4.1,4.3; 424/450; 428/402.2		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
U.S. CL.	264/4.1,4.3; 424/450; 428/402.2	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> *		
Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
Y	US, A, 4,737,323 (MARTIN ET AL.) 12 April 1988, See Fig. 3; Ex's. II & III; and Col. 4, Line 15-Col. 6, Line 7.	1-19
Y	PCT, A1, W086/00238 (CULLIS ET AL.) 16 January 1986, See Fig. 1A; Ex's. 1,2,6 and 8-10; Table I; Page 12, Lines 12-31; Page 14, Lines 1-17; and page 18, Line 7, Page 20, Line 24.	1-19
Y	US, A, 4,687,551 (FURNEAUX ET AL.) 18 August 1987, See Abstract and Col. 8, Lines 22-64.	1-19
Y	ANOTEC INORGANIC MEMBRANE FILTRATION, MANUFACTURE'S LITERATURE, NEW YORK 1988, See fourth page, first & second paragraphs under "Here's Why".	1-19
Y	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Vol. 55, No. 2, February 1989, (JONES ET AL.) "Comparison of a Niew Inorganic Membrane Filter (Anopore) with a Track-Etched Polycarbonate Membrane Filter (Nucle-pore) for direct counting of Direct counting of Bacteria" pages 529-530, See boldface paragraph on page 529.	1-19
<p>* Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"d" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
28 JANUARY 1992	03 MAR 1992	
International Searching Authority	Signature of Authorized Officer:	
ISA/US	RICHARD D. LOVERING	